THE DEVELOPMENT OF EGGS OF THE SCREW-WORM FLY COCHLIOMYIA HOMINIVORAX (COQUEREL) (DIPTERA: CALLIPHORIDAE) TO THE BLASTODERM STAGE AS SEEN IN WHOLE-MOUNT PREPARATIONS

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In studying effects of radiation or chemical mutagens on insect germ cells, being able to examine the chromosomes of nuclei in freshly deposited eggs would provide many advantages. Meiotic division of the oocytes typically occurs after the eggs are deposited. Thus, both the meiotic nuclei and the mitotic ones of the cleavages or later stage divisions could be studied. In addition, only in freshly laid eggs can the chromosomes of sperm be directly examined after treatment that produces dominant lethal mutations. In spite of these advantages, only a few such studies have been made of the nuclei of very young eggs, to ascertain the presence of chromosomal aberrations or abnormal nuclear divisions and death (*i.e.,* Sonnenblick, 1940, on *Drosophila*; Whiting, 1945a, 1945b, von Borstel, 1955, on *Habrobracon*). Neglect of this kind of study has probably been due in part to technical difficulties in collecting and preparing enough eggs at the exact stage of development required. In addition, nuclei and chromosomes of such eggs, as a rule, are very small and thus difficult to study.

Recently the author and his associates became interested in determining whether chromosome aberrations induced in the sperm or oocytes of screw-worms, *Cochliomyia hominivorax* (Coquerel), could be studied in the young egg, in at least a reasonably satisfactory manner. Sectioned material was quickly found unsatisfactory for this purpose and emphasis was placed on whole-mount preparations. Normal development to the stage of blastoderm formation, as followed in the whole-mounts, is described herein. Chromosome aberrations actually found in young eggs after treatment of screw-worm flies with gamma radiation and the alkylating agent, tretamine, were discussed in an earlier paper (LaChance and Riemann, 1964).

Materials and Methods

The sexually mature females of the Florida Normal strain used in this study usually oviposit readily when offered warm lean meat. Each female will then normally produce 200–300 eggs within a period of 15–20 minutes. These eggs, cemented together in a compact mass, are each about 0.7 mm. long and no wider than 0.16 mm. Each one is covered with a rather thick chorion which must be removed in making a whole-mount preparation.

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To obtain eggs of a fairly uniform age, individual flies were allowed to oviposit for one minute in shell vials placed in a water bath heated to 35° C. The flies were then discarded and the vials with the deposited eggs, usually 10 to 20 in number, were removed from the water bath and held at room temperature (24–26° C.) until fixation.

The whole-mount procedure was essentially that described by von Borstel and Lindsley (1959) in their modification of the technique developed by Schmuck and Metz (1931). First, the eggs were dechorionated by shaking them gently in 1% sodium hypochlorite (commercial bleach diluted 1:5 in distilled water) for not more than two minutes. By the end of this period most eggs had lost their chorions. The eggs were removed from the hypochlorite solution by straining them through a cloth. They were then washed briefly in distilled water and placed in rows on 22-mm. coverslips by means of an artist's brush. Each egg was then gently punctured (usually at the posterior or blunt end) with a fine needle so that a thin stream of ooplasm flowed onto the surface of the coverslip. This puncturing was essential for good fixation and the extruded ooplasm served to attach the eggs firmly to the coverslips. As soon as possible after puncturing, the coverslip with its attached eggs was placed in Kahle's fixative. All fluids used in preparing the eggs were kept at room temperature.

It took about 4–5 minutes to handle 10–15 eggs, from the begintning of dechorionation to placing the eggs in the fixative. Fewer eggs could be handled somewhat faster. In general, different groups of eggs fixed at comparable times after deposition were quite similar in stage of development. Also, puncturing one end of the egg or the other, or even allowing the nuclei to flow out of the egg, made little difference in the timing of the stages of development, since fixation occurred rapidly.

After fixation, the eggs were stained according to the Feulgen procedure with Schiff's solution, as outlined by von Borstel and Lindsley. Because of the eggs' rather considerable thickness, the stained and dehydrated specimens were usually mounted on another coverslip instead of on a slide. In this way the mounts could be easily turned over to permit examination of both sides under high magnification. Generally the preparations were allowed to clear for a few days in the mounting medium (Diaphane) before they were examined. This was particularly desirable for examining cleavage nuclei. An optical system that produced little contrast was also found to be highly desirable for examining eggs.

More than 600 eggs were examined in preparing this description of egg development in the screw-worm. Two series of eggs fixed at 15-minute intervals through the first two hours of development and one series fixed at 5-minute intervals from the first to the third hour were prepared. Also prepared were numerous other series of eggs less than one hour old, particularly less than 25 minutes old, since this time interval covered the most significant periods in determining the effects of mutagens on germ cells.

All drawings were made with a camera lucida. However, to save space, some nuclei were drawn somewhat closer together than they would have appeared in true scale.

Observations and Discussions

LaChance and Leverich (1962) demonstrated that the meiotic nuclei of screw-worm eggs go through prophase I and metaphase I while the eggs are still in the

ovaries. By the time the eggs have reached maturity in the 5-day-old females, the oocytes are in early anaphase I, in which stage they remain until the eggs are actually deposited. The chromosomes (six pairs) of these anaphase nuclei are much contracted, and each meiotic figure appears to be merely a single, very small chromatin mass in which some of the homologous chromosomes probably remain in contact with each other. Each nucleus is located in the superficial ooplasm at a point on the dorsal surface of the egg about one-fifth of the total egg length from the anterior end.

In a few eggs, fixed individually as early as three minutes after deposition, the nuclei were still in the condensed early anaphase characteristic of the mature ovarian eggs. Nuclei 1–2 minutes older had longer chromosomes and were usually in late anaphase or telophase I (Fig. 1). After telophase I, the egg nuclei went at once, without any interphase stage, through the second meiotic division, which was completed in 7–8 minutes. At the end of telophase II (Fig. 2), all egg nuclei had formed a straight line that extended from the site of the original anaphase I nucleus to a point somewhat further into the interior of the egg but still near the surface. The position of the line of nuclei (and hence the division plane at anaphase I) with regard to the long axis of the eggs varied widely from egg to egg. In some eggs it extended posteriorly, in others anteriorly, and in still others in various intermediate positions, although always with one end located further in the ooplasm than the other.

The chromosomes of the meiotic nuclei were extremely small and as a rule individual ones could not be recognized, even in the anaphase figures where they were usually fairly well separated.

Immediately after completion of the second division, the terminal nucleus (the one farthest inside the egg) swings out of line and moves as the female pronucleus to a central position in the egg interior at about the same level on the long axis of the egg as the original oocyte. As the female pronucleus moves, it is transformed into an interphase nucleus surrounded by a membrane. The other three meiotic nuclei remain in their original positions as the polar bodies. However, they quickly follow the pronucleus into interphase. This transition starts in the other terminal nucleus a little before it does in the two medial ones.

During the period when the meiotic divisions are taking place, the heads of such sperm as may be present (from 1–5), are seen as short Feulgen-positive rods (Figs. 1 and 2), usually located in the deeper ooplasm of the same general level as the oocyte nuclei. Occasionally a more elongate, threadlike sperm head, such as normal motile sperm possess, was noted, but usually only the short rods were seen, even in the youngest eggs. Sperm tails were presumably present in the eggs but they did not stain and could not be detected.

In near synchrony with the oocyte nuclei, all sperm heads in an egg were transformed into interphase nuclei. One of these nuclei moved to a position immediately adjacent to the female pronucleus to become the male pronucleus. Thus, by the end of about 9 minutes, only interphase nuclei were present in the eggs. When first formed, these nuclei have a diameter of about 5½ microns which increases to 12 microns. Presumably during this 9-minute period cell synthesis takes place, involving among other things DNA replication.

By the end of about 12 minutes, all nuclei, including the polar bodies and any

extra sperm nuclei that might be present, have entered into early prophase (Fig. 3). By 14–15 minutes they have usually reached metaphase with the two pronuclei being included in a single unit to complete syngamy. The first cleavage division (Fig. 4) quickly follows and succeeding cleavages occur in rapid succession (Fig.

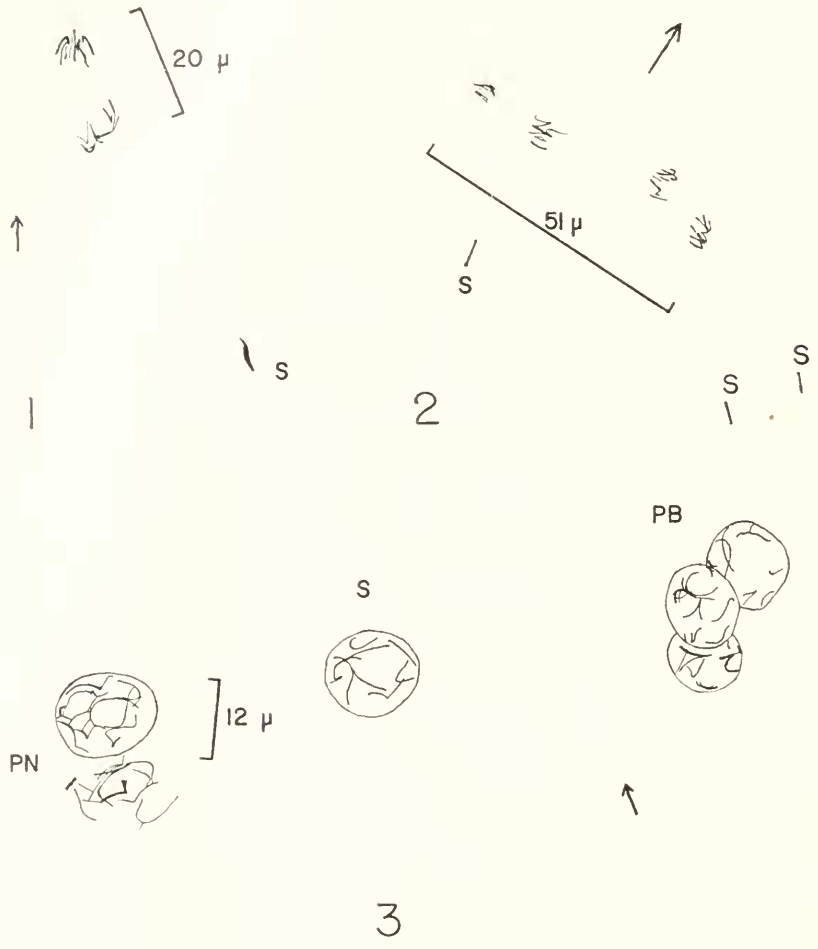

FIGURE 1. Four–5-minute egg, telophase I.
FIGURE 2. Seven–8-minute egg, telophase II.
FIGURE 3. Eleven–12-minute egg, late prophase before syngamy. The nuclear membrane had disappeared from one of the pronuclei.

5). Approximately 5 minutes separate the first few cleavages, but the others occur somewhat less rapidly. By the end of one hour the eighth and final internal cleavage division is usually in progress. Thus, at 25°–26° C. cleavage within the interior of the egg occupies a period of about 45 minutes, with the average division cycle lasting only about 6 minutes. This rate is somewhat faster than the cleavage cycle of about 9–10 minutes at 20°–30° C. reported for *Drosophila* (Sonnenblick, 1950) and 10

minutes at 20° C. for *Calliphora vicina* (R. and D.) [*erythrocephala* (Meigen)] (Melander, 1963) and may represent the most rapid rate of mitosis reported for multicellular animals (Mazia, 1961), although it would seem rather probable that other higher Diptera develop as rapidly.

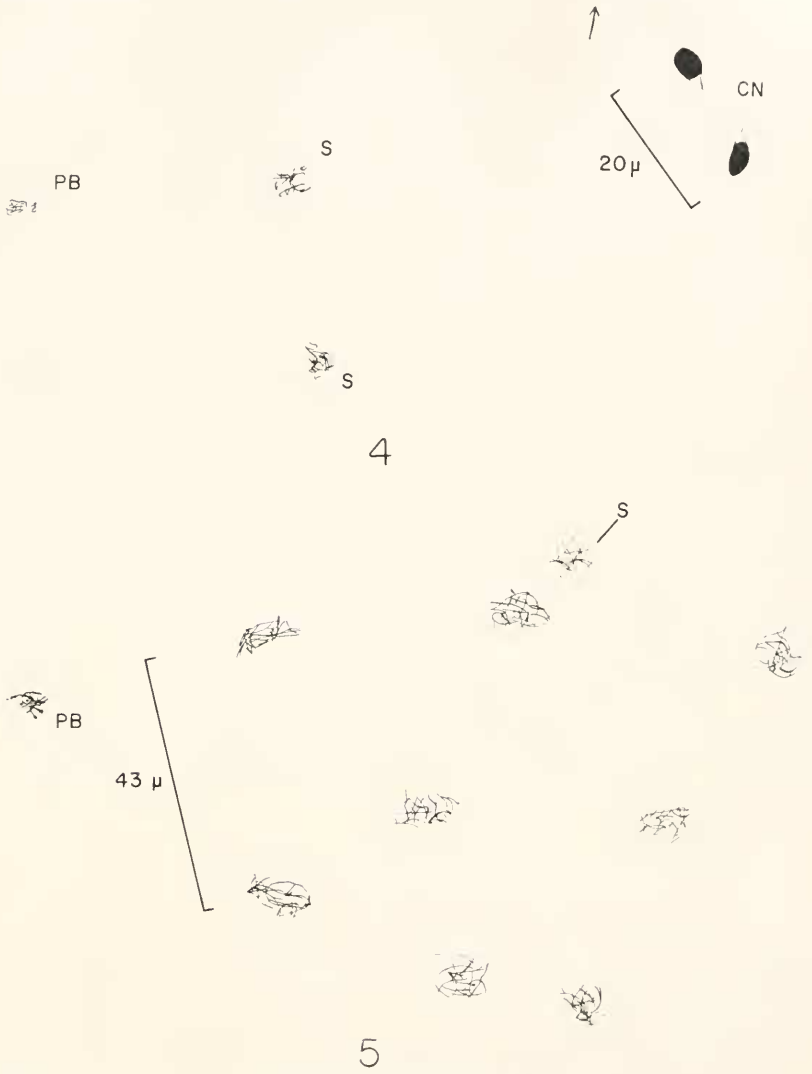

FIGURE 4. Fifteen–16-minute egg, first cleavage telophase.
FIGURE 5. Thirty–31-minute egg, fourth cleavage metaphase.

Observing cleavage nuclei was rather less satisfactory than observing meiotic ones. In part, the difference was due to the obstruction caused by the overlying ooplasm even after it had cleared, but in addition the cleavage nuclei appeared to stain less deeply. Again, individual chromosomes could not be distinguished as a

rule. Sometimes male and female chromosome complements remained somewhat separated during the first cleavage division, but more usually they could not be recognized after they had formed the first metaphase figure. Viewing cleavage divisions was made easier by the fact that they all occurred in a plane rather closely parallel to the egg surface.

The first cleavage nuclei remain in the area in which syngamy occurs. With repeated divisions there is a spread of nuclei, and by the end of the eighth division they are rather evenly distributed along the length of the egg.

The somewhat slower velocity of the later internal cleavages appeared to be associated with a change in duration of the different stages of cell division. Very careful timing was required to obtain metaphase, anaphase, or telophase figures during the first 2–3 cleavage divisions. On the other hand these stages were found more often than interphases or prophases in eggs fixed at various later times. All the first 8 cleavages are probably completely synchronous in the intact egg, but in the usual stained specimen a slight gradient of development away from the site of puncturing was noted.

After the end of the eighth cleavage division in the interior of the egg, most nuclei have migrated near the surface of the egg to form the incipient blastoderm or blastema by the end of 1 hour and 10 minutes. After the blastema is first formed, four other cleavage divisions of most of the nuclei occur. The first of these divisions takes place shortly after the nuclei complete their movement to the egg surface. The last division is usually underway or completed by the end of the second hour. This cycle, lasting about 15 minutes, compares rather closely with the 17-minute cycle that Agrell (1963) and Melander (1963) reported for the same four divisions in *C. vicina*. After the 12th division a prolonged interphase takes place, at the end of which further divisions occur. During the long interphase period, cell membranes are formed around the nuclei located in the surface ooplasm, to become the definitive blastoderm.

These last four cleavages are not completely synchronous like the first 8, but instead appear to follow the pattern reported by Agrell (1963) for *C. vicina*. For the first three divisions there was a rather slight mitotic gradient from the anterior end of the egg, and also during the 11th division some nuclei near the posterior end were observed to divide earlier than those more anterior. During the 12th division a definite mitotic gradient proceeding from both ends of the egg was noted (Fig. 6).

Not all of the early cleavage nuclei migrate to the surface ooplasm to form the blastema. Some remain behind as the so-called yolk nuclei or vitellophags. These divide out of synchrony with the blastema nuclei, to form rather massive clusters of nuclei (Fig. 7) by the time the 12th division is completed. No evidence was seen to indicate that any of the blastema nuclei migrate inward to increase the number of yolk nuclei.

Shortly before the 10th cleavage a cluster of nuclei appear outside of the blastema at the posterior end of the egg. These are the so-called pole cells which presumably, here as in other species, include the primordial germ cells. In *C. vicina* these cells (actually only nuclei) are also set off at about the time of the 10th division, although, according to Sonnenblick (1950), the first ones appear at the time of the 9th division in *Drosophila*. No divisions were detected among the pole cell nuclei during the periods when the later cleavages occurred.

Melander (1963) described in great detail how the "pseudo-chiasmata," formed during the 9–13th nuclear divisions in *C. vicina,* result in chromosome diminution by causing the loss of small chromosome fragments. No attempt was made to study possible chrosome diminution in the screw-worm. However, in the blastema

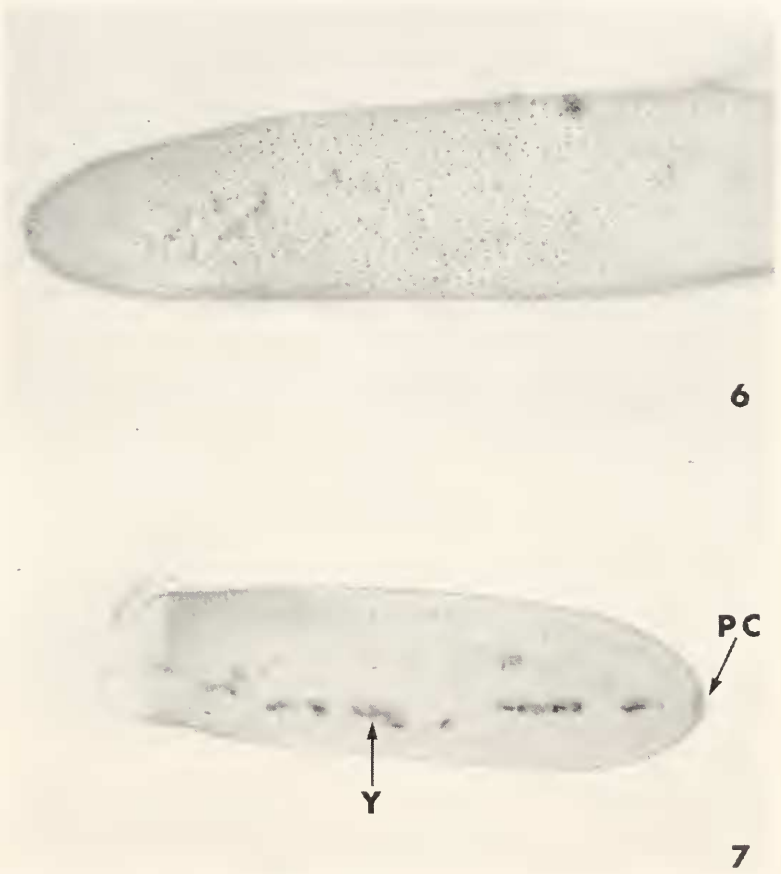

FIGURE 6. One hour and 59–60-minute egg. The nuclei at either end have gone into interphase while those in the central region are still dividing; 400 ×.

FIGURE 7. Two hour and 15–16-minute egg, showing the clumped yolk cells and the interphase nuclei of the blastema after the completion of the 12th division; 320 ×.

Explanation of captions: C N, cleavage nuclei; P B, polar bodies; P C, pole cells; P N, pronucleus; S, sperm cell; Y, yolk cells.

many anaphases exhibited configurations that appeared to be similar to the pseudo-chiasmata described by Melander. Thus, it seems probable that the events in the two species are at least somewhat similar.

As stated earlier, the polar bodies and surplus sperm nuclei of screw-worm eggs go into metaphase at the same time as the pronuclei and remain in this stage until they eventually disappear. The sperm nuclei can be seen during early cleavages

TABLE I

*Time sequence in the development of screw-worm eggs through blastoderm formation**

Minutes	Stage of development	Minutes	Stage of development
0–4	Sperm move into area of oocyte which remains in early meiotic anaphase I.	44–46	Cleavage V completed and division VI may be in progress; 32 nuclei.
4–6	Meiotic division I is completed.	59–61	Cleavage VIII generally in progress; 128 dividing nuclei.
5–8	Meiotic division II is completed.	74–76	Blastema formed with division IX usually in progress.
8–12	All nuclei go into interphase. Pronuclei move to adjacent position in the interior of the egg.	90–91	Division X in progress. Pole cell nuclei set aside.
12–13	All nuclei pass synchronously into metaphase. Syngamy occurs as the two pronuclei form a single figure.	104–106	Division XI usually in progress.
14–16	Completion of cleavage I.	120–122	Division XII usually in progress.
29–31	Cleavage III in progress or completed, 4–8 nuclei.	150	Cell membranes around surface nuclei, definitive blastoderm.

* Oviposition at 35° C., development after one minute at 24–26° C.

but not during the later ones. The polar bodies remain until the blastema is formed but then quickly undergo dissolution. Very rarely we observed one of the haploid polar bodies dividing at the same time as the first cleavage nucleus.

2. Polyspermy in screw-worm eggs

It has long been considered a general rule that in insects each egg is penetrated by several sperm (Wigglesworth, 1950). However, Hildreth and Lucchesi (1963) found that, contrary to the observations of earlier workers, the eggs of *Drosophila melanogaster* and *D. virilis* usually received only a single sperm. As a result of their studies, they raised the question as to how common polyspermy might actually be in insects.

Many screw-worm eggs contained only a single sperm, but more often than not they had two or more sperm. In no egg, however, were more than 5 sperm found and this number was quite unusual. Also, an inseminated fly often deposited a few eggs that had not been fertilized. The presence of these unfertilized eggs probably explains why somewhat less than 100% of the eggs from normally inseminated females usually hatch.

TABLE II

Distribution of sperm found in a sample of screw-worm eggs

Unfertilized eggs	2
Eggs containing one sperm	30
Eggs containing two sperm	39
Eggs containing three sperm	14
Eggs containing four sperm	3
Total number of eggs	88
Total number of sperm	162

The distribution of sperm in 88 eggs from 10 different females is shown in Table II. All of these eggs were fixed before they were 8 minutes old, when the rodlike sperm heads are relatively easy to count.

Occasionally a sperm nucleus was observed very near one of the polar bodies although in no instance had a second zygote actually been formed. However, the appearance of an occasional gynandromorph probably indicates that a second zygote is sometimes formed.

3. *Development of unfertilized eggs*

Virgin screw-worm females oviposit nearly as readily as inseminated ones, but their eggs apparently never hatch. However, young eggs in which no sperm could be detected often completed both meiotic divisions. To determine how far development advances in unfertilized eggs, whole-mounts of 8–20 eggs were prepared from each of 15 virgin flies. One portion of the eggs were fixed at 5½–7 minutes after

TABLE III

The development of eggs from virgin females

	Age at fixation		
	5½–7 min.	14–16 min.	1¼–2 hrs.
No. of females	5	6	4
No. of eggs	53	72	60
Aborted meiotic division I	12		
Meiotic division I completed or in progress	19		
Meiotic division II completed or in progress	22		
Pronucleus not formed or did not migrate to interior of egg		35	33
Normal migration of pronucleus		33	24
Cleavage I completed or in progress		4	3
Cleavage II completed or in progress		0	0

deposition, another portion at 14–16 minutes, and a third at 1¼–2 hours. To eliminate any possibility that dechorionation and other steps involved in the handling of the eggs might initiate development, these operations were limited to not more than 5 minutes immediately prior to fixation.

Results of observations on the stained preparations are shown in Table III. They demonstrate that fertilization is not strictly necessary for initiation of development. However, it is also evident that the presence of sperm in the eggs has some influence even on meiotic divisions, for nearly one-third of the nuclei fixed at 5½–7 minutes had failed to complete the first division. It was also obvious that some of the other nuclei could not have gone through the second division. Even in eggs in which the two divisions were completed, the pronuclei had often failed to migrate into the interior of the egg. Thus, in over half the eggs fixed at 14 minutes or later, clumps of chromatin were found only at the site normally occupied by the three polar bodies. In some eggs, each of these clumps was observed to be composed of four smaller clumps representing all of the meiotic nuclei. Observations on other unfertilized eggs fixed at 8–9 minutes demonstrated that when meiosis was com-

pleted, the oocyte nuclei went through the usual interphase stage before returning to metaphase.

Scoring of the older unfertilized eggs was easier because development always stopped at a stage at which all nuclei were in metaphase. Apparently no nuclei had disappeared by the time the oldest preparations were fixed.

The author wishes to express his appreciation to Dr. Leo LaChance, who suggested this study and helped in many ways during its progress. He also wishes to thank Miss Ann Leverich and Miss Sarah Bruns for their technical assistance.

Summary

1. Development of screw-worm eggs from the first meiotic division to blastoderm formation was studied from whole-mount preparations. Both meiotic divisions were completed by 7–8 minutes. Syngamy at 14–15 minutes was quickly followed by the first cleavage division. The first 8 cleavages took place within the interior of the eggs, and the last of these occurred approximately one hour after egg deposition. After the 8th division, most cleavage nuclei moved near the egg surface to form the blastema by the end of about 1 hour and 10 minutes. This movement was followed by four more divisions of the blastema nuclei. The last of these divisions was underway, or had been completed, by the end of the second hour. There was then a prolonged interphase period, during which cell membranes formed around the blastema nuclei to become the definitive blastoderm.

2. The first 8 cleavages were synchronous, but for the last four divisions an anterior-posterior gradient was evident. During the 11th division, and especially during the 12th division, there was also an accompanying mitotic gradient proceeding from the posterior end of the egg.

3. After the 8th cleavage some nuclei remained behind to form the yolk nuclei or vitellophags. Also, the pole cells were set aside after the 9th division but prior to the 10th.

4. A low order of polyspermy was found in most eggs, but many of them received only a single sperm. Most unfertilized eggs completed at least the first meiotic division, but very few of them achieved the first cleavage division and none developed further than this stage.

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